418 Rec'd PCT/PTO 20 APR 1999

FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

APR 20 1999 PATENT & TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: BMID9913US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): ~~09/284786~~ 09/284787

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP97/05783	20 October 1997	21 October 1996

TITLE OF INVENTION

MONOCLONAL ANTIBODIES AGAINST THE EPITOPE YPYDVPDYA, A PROCESS FOR PRODUCING THE SAME AND THEIR USE

APPLICANT(S) FOR DO/EO/US

Thomas EMRICH, Matthias HINZPETER and Michael GROL

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Sequence Listing in German (3pp);
General Appointment of Representative for U.S. Patent and Patent Application (1pp); and
Return postcard.

PCTUS1/REV03

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
	PCT/EP97/05783	BMID9913US

20. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Search Report has been prepared by the EPO or JPO	**$930.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$720.00**
☐	No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	**$790.00**
☒	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	**$1,070.00**
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	**$98.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$1,070.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	11 - 20 =	0	x $22.00	**$0.00**	
Independent claims	3 - 3 =	0	x $82.00	**$0.00**	
Multiple Dependent Claims **(check if applicable).**			☒	**$270.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,470.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☐				**$0.00**	
SUBTOTAL =				**$1,470.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,470.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,470.00**	
				Amount to be: refunded	$
				charged	$

☐ A check in the amount of to cover the above fees is enclosed.

☒ Please charge my Deposit Account No. **50-0877** in the amount of **$1,470.00** to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **50-0877** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Marilyn L. Amick
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Marilyn L. Amick
SIGNATURE

Marilyn L. Amick
NAME

30,444
REGISTRATION NUMBER

April 20, 1999
DATE

418 Rec'd PCT/PTO 20 APR 1999

TRANSMITTAL LETTER TO THE UNITED STATES RECEIVING OFFICE

PTO-1382 (Rev. 4-1995) PCTUS2.FRM /REV00

Date	April 20, 1999
International Application No.	
Attorney Docket No.	BMID9913US

I. **Certification under 37 CFR 1.10 (if applicable)**

EL315019987US	April 21, 1999
Express Mail mailing number	Date of Deposit

I hereby certify that the application/correspondence attached hereto is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Assistant Commissioner for Patents, Washington, D.C. 20231.

Rose Edwards	Rose Edwards
Signature of person mailing correspondence	Typed or printed name of person mailing correspondence

MJ 09/284786 PCT

09/284787

II. ☐ **New International Application**

TITLE		Earliest priority date (Day/Month/Year)

SCREENING DISCLOSURE INFORMATION: In order to assist in screening the accompanying international application for purposes of determining whether a license for foreign transmittal should and could be granted and for other purposes, the following information is supplied. (Note: check as many boxes as apply):

A. ☐ The invention disclosed was **not** made in the United States.

B. ☐ There is no prior U.S. application relating to this invention.

C. ☐ The following prior U.S. application(s) contain subject matter which is related to the invention disclosed in the attached international application. *(NOTE: priority to these applications may or may not be claimed on form PCT/RO/101 (Request) and this listing does not constitute a claim for priority).*

application no.		filed on	
application no.		filed on	

D. ☐ The present international application ☐ is identical ☐ contains less subject matter than that found in the prior U.S. application(s) identified in paragraph C.

E. ☐ The present international application ☐ contains additional subject matter not found in the prior U.S. application(s) identified in paragraph C. above. The additional subject matter is found on pages ____ and ☐ **DOES NOT ALTER** ☐ **MIGHT BE CONSIDERED TO ALTER** the general nature of the invention in a manner which would require the U.S. application to have been made available for inspection by the appropriate defense agencies under 35 U.S.C. 181 and 37 CFR 5.1. See 37 CFR 5.15

III. ☐ **A Response to an Invitation from the RO/US.** The following document(s) is (are) enclosed:

A. ☐ A Request for An Extension of Time to File a Response

B. ☐ A Power of Attorney (General or Regular)

C. ☐ Replacement pages:

pages		of the request (PCT/RO/101)	pages		of the figures
pages		of the description	pages		of the abstract
pages		of the claims			

D. ☐ Submission of Priority Documents

Priority document		Priority document	

E. ☐ Fees as specified on attached Fee Calculation sheet form PCT/RO/101 annex

IV. ☐ **A Request for Rectification under PCT 91** ☐ **A Petition** ☐ **A Sequence Listing Diskette**

V. ☒ **Other** (please specify): Transmittal Letter to the United States Designated/Elected Office (DO/EO/US) Concerning a Filing Under 35 U.S.C. 371

The person signing this form is the:	☐ Applicant	Marilyn L. Amick
	☒ Attorney/Agent (Reg. No.) 30,444	Typed name of signer
		Marilyn L. Amick

Attorney Docket No. BMID9913US PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Thomas EMRICH, *et al.*

Application No.: To Be Assigned Group No.: To Be Assigned

Filed: April 20, 1999 Examiner: To Be Assigned

For: Monoclonal Antibodies Against The Epitope YPYDVPDYA, A Process For Producing The Same And Their Use

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Preliminary to examination of the captioned application, Applicants respectfully request entry of the following.

In the Claims

Cancel claims 1-9.

Add new claims 10-17 as follows:

10. A monoclonal antibody against the epitope YPYDVPDYA, said antibody being derived from the haemagglutinin of the human influenza virus, or fragments thereof, and having an affinity of $> 10^8\ M^{-1}$.

11. A monoclonal antibody against the epitope YPYDVPDYA, said antibody being derived from the haemagglutinin of the human influenza virus, or fragments thereof, and having an affinity of $10^9 - 10^{10}\ M^{-1}$.

12. The monoclonal antibody of claim 1 or claim 2, wherein said antibody is produced by hybridomas which are obtained by fusing mouse P3x63-Ag8.653 myeloma cells with B lymphocytes from Lou/C rats, said Lou/C rats having been immunized with a haemagglutinin peptide.

13. The monoclonal antibody of claim 1 or claim 2, wherein said antibody is produced by hybridomas which are obtained by fusing mouse P3x63-Ag8.653 myeloma cells with B lymphocytes from Lou/C rats, said Lou/C rats having been immunized with a haemagglutinin peptide, wherein said immunization is carried out with a haemagglutinin peptide coupled to keyhole limpet haemocyanin.

14. The monoclonal antibody of claim 1 or claim 2, wherein said antibody is produced by hybridoma R 3A12 deposited at the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" under the No. DSM ACC2286 (08.10.1996).

15. A method for the production of a monoclonal antibody against the epitope YPYDVPDYA comprising:
(a) synthesizing a haemagglutinin peptide,
(b) immunizing a small mammal with said peptide,
(c) isolating B lymphocytes from the spleen of said mammal and fusing said lymphocytes with mouse P3x63-Ag8.653 myeloma cells to form clones,
(d) selecting clones formed in step (c) which bind to a haemagglutinin peptide and to a haemagglutinin fusion protein, and
(e) selecting a clone with a high affinity from those selected in step (d) and establishing said clone as a hybrid cell line.

16. The method of claim 6, wherein said haemagglutinin peptide is selected from the group consisting of acetyl-**YPYDVPDYA**GSGSK (ε-biotinoyl) amide and biotinoyl-ε-Aca-SGSG**YPYDVPDYA** amide.

17. The method of claim 6, wherein said haemagglutinin fusion protein is haemagglutinin-tagged glutathione-S-transferase.

Respectfully submitted,

Date: April 20, 1999

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DECLARATION

I, Sabine Frieda Katharina Town, declare that I am a citizen of the Federal Republic of Germany, residing at Waldstraße 45, 82386 Oberhausen, Federal Republic of Germany, that I am fluent in German and English, that I am a competent translator from German into English and that the attached is a true and accurate translation made by me into the English language of International Patent Application No. PCT/EP97/05783.

I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

I hereby subscribe my name to the foregoing declaration, this fourth day of March 1999.

Sabine F.K. Town

Monoclonal antibodies against the epitope YPYDVPDYA, a process for their production and their use

The invention concerns monoclonal antibodies against the epitope YPYDVPDYA which is derived from the haemagglutinin of the human influenza virus and are suitable for the detection and isolation of native haemagglutinin from the human influenza virus, of modified haemagglutinin or of haemagglutinin fusion proteins and have an affinity of > 10^8 M^{-1}, in particular of 10^9 to 10^{10} M^{-1}.

Haemagglutinins are substances - usually glycoproteins - which have the ability to agglutinate erythrocytes. They occur among others as components of viruses such as e.g. in myxoviruses or pox viruses. The haemagglutinin (HA) of the influenza virus which is a membrane-coated virus with a (-) RNA genome is of particular importance. The influenza haemagglutinin is a transmembrane surface antigen which protrudes from the spherical lipid coat in the form of spikes which can be seen in electron-microscopic pictures. The HA spikes are trimers, the monomers of which are composed of two polypeptide chains, HA1 (46,000-65,000 D) and HA2 (21,000-30,000 D). The haemagglutinin in the membrane of the influenza virus enables the virus to penetrate into susceptible host cells e.g. of the respiratory tract.

It is known that antibodies against haemagglutinin are effective inhibitors of a viral infection. However, the specific affinity of the antibody does not generally

apply to the whole macromolecular protein but only to a special epitope.

Nowadays the technique of epitope tagging, i.e. the attachment of an epitope to a protein by molecular biological techniques, is a method that is frequently used to analyse proteins and in principle is independent of the epitope used. In this method the primary sequence of an arbitrary protein is extended by a few amino acids with the aid of recombinant techniques. The decisive factor is only the presence of a specific and high affinity antibody with a known recognition sequence. Using antibodies that are specifically directed against the extended part of the protein, this method enables for example the analysis of the molecular weight of the protein, its cellular location, post-translational modifications or interactions with other factors without requiring the presence of protein-specific antibodies.

An advantage of viral epitopes compared to cellular epitopes is that these protein sequences do not usually occur in bacterial and eukaryotic proteins and thus no cross-reactions would be expected in bacterial or cellular systems.

A viral epitope frequently described in the literature that is used for such analyses is derived from the haemagglutinin of the human influenza virus. This epitope has the amino acid sequence YPYDVPDYA (98 - 106) (Field, J. et al. (1988), Mol. Cell. Biol. Vol. 8, No. 5, 2159-2165 and Wilson et al., Cell 37, 767 - 778, (1984)). Monoclonal antibodies (mAB) against this epitope have been described and are available such as for example the mAB 12CA5 (P.A. Kolodziej and Young,

R.A., Meth. Enzymol. (1991), Vol. 194, 508-519; Chen, Y.-T. et al. (1993), Proc. Natl. Acad. Sci., Vol. 90, 6508-6512)) and the anti-HA-BabCo.

However, a disadvantage of these antibodies is that their affinity is not high enough and therefore the epitope-specific antibodies have to be used in a high concentration for a sensitive detection of the corresponding fusion proteins which can lead to unspecific interactions which for example become apparent as cross-reactions in a Western blot (cf. Chen et al., p. 6510).

The inadequate affinity also leads to a lower sensitivity of the known anti-HA mAB.

Therefore the object of the invention was to provide monoclonal antibodies against the viral epitope YPYDVPDYA which have a higher affinity and which are thus suitable for highly sensitive haemagglutinin tests or HA fusion protein tests and give reproducible results.

According to the invention monoclonal antibodies are provided which recognize the epitope having the amino acid sequence YPYDVPDYA (98 - 106) of the haemagglutinin of the human influenza virus as well as corresponding fragments thereof and have an affinity of $> 10^8$ M^{-1}, in particular of 10^9 to 10^{10} M^{-1}. In this connection epitope fragments are understood in particular as those amino acid sequences which correspond to at least 70 % of the sequence YPYDVPDYA or are shortened by at least one to two terminal amino acids.

In order to produce the monoclonal antibodies, small mammals, preferably rats, such as e.g. Lou/C rats or mice such as e.g. BalbC mice or rabbits are immunized with a HA peptide synthesized by standard methods. An uncoupled HA peptide or a HA peptide which is optionally coupled N-terminally or C-terminally to a carrier protein or a HA fusion protein is used as the antigen. Keyhole limpet haemocyanin (KLH) or bovine serum albumin (BSA) were preferably used as carrier proteins. Subsequently B lymphocytes were isolated from the spleen of the animals and immortalized by cell fusion with suitable myeloma cells or by other known methods such as e.g. by means of oncogenes (Jonak, Z.L. et.al., (1988) Adv. Drug Rev. 2:207-228) or in an electrical field (Zimmermann, U. (1982), Biochim. Biophys. Acta 694:227-277). The cell fusion was preferably carried out according to the invention with spleen cells of Lou/C rats and myeloma cells from the mouse line P3x63-Ag8,653 (Kearney, J.F. et al (1979), J. Immunol. 123, 1548-1550).

In this process the lymphocytes and the myeloma cells are fused by known methods, in particular by polyethylene glycol fusion (PEG), virus fusion or electrofusion and the hybrid cells (cell clones) that are formed are also selected by known methods such as e.g. by using selection media.

Thus for example positive clones were firstly tested with HA peptides and then with HA fusion proteins. In a first screen a biotinylated HA peptide e.g. Bio-C-HA (acetyl-**YPYDVPDYA**GSGSK (ε-biotinoyl)-amide) or Bio-N-HA (biotinoyl-ε-Aca-SGSG**YPYDVPDYA**-amide) was used and a HA-tagged glutathione-S-transferase (GST) was used in a second screen. Clones that were again positive were

subsequently examined with regard to their affinity with the aid of plasmon resonance in a BiaCore system and they were selected.

The hybrid cells were cloned, cultured and multiplied according to known methods and optionally stored in liquid nitrogen.

The cell lines R 3F10, R 3A12 and R 6D12 were established as the most active cell clones with a stable antibody production. The hybridoma R 3A12 was deposited on the 08.10.1996 at the "Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ)", Mascheroder Weg 1b, 38124 Braunschweig under the number DSM ACC2286.

For the antibody isolation the hybrid cells were further propagated in cell culture or optionally in vivo by transplantation as ascites tumours. The mABs were isolated from the cell culture supernatants or optionally from the ascites fluid of the tumour-carrying experimental animals.

The mAB produced in high concentration by the hybrid cells which are characterized by an excellent specificity and binding strength for the YPYDVPDYA epitope of the haemagglutinin of the human influenza virus or for corresponding epitope fragments are obtained according to the invention. They enable the highly sensitive detection and isolation of haemagglutinin as well as of proteins to which the HA epitope YPYDVPDYA has been attached.

The affinity of the mAB according to the invention is $> 10^8\ M^{-1}$. Thus the affinity of the mAB 3F10 at $10^{10}\ M^{-1}$

is approximately 30-fold higher than that of the known antibodies 12CA5 (10^8 M^{-1}) and BabCo (10^7 M^{-1}).
The affinity of the mABs 3A12 and 6D12 according to the invention is 10^9 M^{-1} and is thus also higher than that of the known antibodies. The mABs according to the invention can be used in much lower concentrations and cross-reactions can be almost completely ruled out. They enable an improved sensitivity of the detection. It has turned out that they recognize native HA of the influenza virus, modified HA as well as HA fusion proteins. Hence they can be used very well for the determination of proteins in known detection reactions such as e.g. a solid phase two-side binding test.

Figure legends

<u>Figure 1:</u>
Affinities of the mABs according to the invention compared to the mAB 12CA5 and anti-HA BabCo.

<u>Figure 2:</u>
Immunoblot analysis of a HA-modified glutathione-S-transferase protein with a mAB (clone 3F10) according to the invention and anti-HA of the prior art (clone 12CA5); a) detection with anti-rat-peroxidase, b) anti-rat biotin/streptavidin-peroxidase.

<u>Figure 3:</u>
Immunoblot analysis of a HA-modified glutathione-S-transferase protein with a mAB (clone 3F10) according to the invention and anti-HA of the prior art (clone 12CA5).

Figure 4:
Immunoblot analysis of a HA-modified glutathione-S-transferase protein with enzyme (peroxidase) conjugates of an antibody according to the invention (clone 3F10) and an antibody of the prior art (clone 12CA5).

Figure 5:
Immunoprecipitation of a HA-modified green fluorescent (GFP-HA) protein with a mAB (clone 3F10) according to the invention and anti-HA of the prior art (clone 12CA5).

Subsequently the invention is further elucidated by the following examples of use.

Example 1:

Production of clones R 3F10, R 3A12 and R 6D12

HA peptide preparation

The following peptides were synthesized:
Bio-C-HA (acetyl-**YPYDVPDYA**GSGSK (ε-biotinoyl)-amide)
Bio-N-HA (biotinoyl-ε-Aca-SGSG**YPYDVPDYA**-amide)
KLH-MPS-CUZU-HA-C
KLH-MPS-CUSU-HA-N

Immunization of small mammals

Lou/C rats were immunized intraperitoneally with KLH-coupled HA peptide according to the following scheme:

For the primary immunization the animals were injected with 50 µl KLH-coupled HA peptide in complete Freund's adjuvant.

Further immunizations were carried out with 50 µg KLH-coupled HA peptide in incomplete Freund's adjuvant.

Fusion

The subsequent fusion of the spleen cells of the Lou/C rats was carried out using mouse P3x63-Ag8,653 in the presence of PEG according to Kremmer et al (1990) Hybridoma 9, 309-317.

Selecting the clones

Screening scheme:

1st Screen

- An SA-coated microtitre plate (MTP) was coated with 1 µg/ml Bio-C-HA or Bio-N-HA,
- hybridoma supernatants were used undiluted and added to the coated MTP,
- the bound antibodies were detected with the aid of anti-rat-POD conjugate/TMB substrate.

2nd Screen

- A maxisorb MTP was coated with HA-tagged GST (1 µl/ml in carbonate buffer),
- the hybridoma supernatants selected in the 1st screen were again used undiluted and added to the coated MTP,
- the bound antibodies were detected with the aid of anti-rat-POD conjugate/TMB substrate.

3rd/4th Screen

- BiaCore measurements were carried out with an analogous coating.

Result

5 clones with the highest affinity and the longest half-time of dissociation were selected with the aid of plasmon resonance in the BiaCore system. They were named R 3F10, R 3A12, R 6D12, R 4H10 and M5B9. The affinity was only slightly different depending on the biotinoylated position of the peptides (C-terminus or N-terminus cf. Fig. 1).

The clones R 3F10, R 3A12 and R 6D12 were established as cell lines. They exhibit a good growth and a stable antibody production, and the antibodies that are produced have a 10-fold to 100-fold higher affinity than the monoclonal antibodies of the prior art 12CA5 and anti-HA BabCo.

Fig. 1 shows the affinities of the mABs according to the invention compared to the mAB 12CA5 and anti-HA BabCo.

Example 2

Determination of the affinity constants as well as of the rate constants of association and dissociation of the antibodies that are produced

The affinity constants and rate constants of association and dissociation of the antibodies that were produced was determined with BIAcore® from the Pharmacia

Biosensor Company (BIA stands for Biospecific Interaction Analysis). The measurement principle is based on surface plasmon resonance. The measurement is carried out on a biosensor, the so-called sensor chip. The biotinylated peptide is coupled to the streptavidin coated sensor chip by means of a non-covalent, high affinity bond. A solution of the antibody to be examined is passed over the sensor chip in the process of which the antibody is bound to the immobilized peptide by means of non-covalent forces of interaction.

The binding of the individual components increases the mass density on the surface of the sensor chip which is converted by the instrument into a proportional measurement signal. The rate constants of association and dissociation and, derived therefrom, the affinity constants can be calculated from the change in the signal versus time, the sensorgram.

The antibody-peptide complexes can be detached again with simple agents without impairing the peptides bound to the surface so that additional binding experiments can be carried out on the same sensor chip under identical boundary conditions.

In order to couple the biotinylated peptides to the sensor chip (SA, Pharmacia Biosensor) a solution containing a concentration of 50 nmol/l in HBS (10 mmol/l HEPES, 150 mmol/l NaCl, 3.4 mmol/l EDTA, 0.05 % P20 pH 7.4) is passed over the sensor chip at a flow rate of 5 ml/min.

Afterwards the antibodies are added in HBS and binding to the peptides is monitored at a flow rate of 10

µl/min. The rate constants of association and dissociation of the binding of the antibody to the peptides are calculated from the sensorgrams with the aid of the manufacturer's software (BIA evaluation 2.1, Pharmacia Biosensor). The affinity constants are calculated from Ka = kon/koff. The values determined in this manner for the antibodies according to the invention to Bio-C-HA and BioN-HA as antigens are summarized in Table 1.

Table 1

anti-HA-mAB	Antigen	kon l/mol*s	koff 1/s	Ka l/mol
Babco	Bio-C-HA	9.5 E +3	1.0 E -3	9.3 E +6
12CA5	Bio-C-HA	2.3 E +4	2.6 E -4	9.2 E +7
R3F10	Bio-C-HA	5.9 E +5	4.8 E -5	1.2 E +10
R3A12	Bio-C-HA	1.7 E +5	5.4 E -4	3.1 E +8
R6D12	Bio-C-HA	4.0 E +5	7.6 E -5	5.2 E +9
Babco	Bio-N-HA	1.2 E +4	5.0 E -4	2.3 E +7
12CA5	Bio-N-HA	5.3 E +4	2.1 E -4	2.6 E +8
R3F10	Bio-N-HA	6.2 E +5	9.1 E -5	6.8 E +9
R3A12	Bio-N-HA	7.7 E +5	1.4 E -4	5.7 E +9
R6D12	Bio-N-HA	5.9 E +5	1-2 E -4	5.1 E +9

Example 3

Comparative immunoblot analysis of a HA-modified protein using an antibody according to the invention and an antibody known from the prior art

3.1 Variation of the antigen

Glutathione-S-transferase modified with the HA epitope (GST-HA) was serially diluted to the stated amounts, separated by means of SDS polyacrylamide gel

electrophoresis and, after transfer onto a nylon membrane, reacted with the stated antibodies at the stated concentrations of the primary antibodies (1 µg/ml clone 12CA5; 0.1 µg/ml clone 3F10; Western blot analysis). Bound anti-HA antibodies were subsequently detected using anti-mouse-peroxidase (in the case of clone 12CA5), anti-rat-peroxidase (in the case of clone 3F10, a)) or anti-rat-biotin/streptavidin peroxidase (in the case of clone 3F10, b)) and by chemiluminescence detection.

SDS polyacrylamide gel electrophoresis

8 x 7 cm gels with a thickness of 0.75 mm were used (BIO-RAD, Mini-Protean II™). For a 15 % gel, 6 ml separation gel solution (1 x) was poured between the plates. The top edge of the separation gel was carefully covered with a layer of 1 ml water. After 30 min polymerisation, the water was removed and it was filled with 2 ml collection gel solution (1 x). The samples were mixed with 1 volume of two-fold concentrated Laemmli buffer, incubated for 10 min at 60°C and pipetted into the rinsed gel pockets. The electrophoresis was carried out in mobile buffer at a current strength of ca. 15 mA.

Solutions:

4 x separation gel solution:

1.5 M Tris/HCl, pH 8.8
0.4 % SDS

4 x collection gel solution:

0.5 M Tris/HCl, pH 6.8
0.4 % SDS

2 x Laemmli buffer:

1.52	g Tris	per 100 ml
10	ml glycerol	pH 6.8 adjusted with 1 N HCl
2.0	g SDS	
2.0	ml 2-mercaptoethanol	
1	mg bromophenol blue	

5 x mobile buffer:

15.1	g Tris	per 1 l
72.0	g glycine	
5.0	g SDS	

Transfer onto nylon membrane

After the electrophoretic separation of the protein samples, a nylon filter corresponding to the gel size and moistened with H_2O was placed on the gel. To ensure better contact two correctly cut Whatman® 3 MM papers were placed on each of the two sides and the construction was clamped in a BIO-RAD electroblot apparatus. After filling the chamber with transfer buffer, the proteins were transferred onto the membrane by applying a voltage of 70 V (current strength, I=250-350 mA) for 45 - 60 min while cooling on ice and stirring.

Solution:

transfer buffer:	25	mM	Tris/HCl, pH 8.3
	192	mM	glycine

Detection

After carefully removing the membrane, the filter was

incubated for 60 min at RT in PBS solution to which 1 % blocking reagent (PBS/PR; Boehringer Mannheim) had been added in order to saturate binding sites that are still free. It was washed twice for 10 min in PBS buffer containing 0.1 % Tween 20 (PBST) and reacted for 60 min at RT with the corresponding anti-HA antibody in the stated concentrations. After washing three times in PBST buffer (5 min each time, RT), the filters were incubated for a further 60 min at room temperature with an anti-species IgG-peroxidase conjugate (anti-rat-peroxidase, 20 mU/ml PBS/BR; anti-mouse-peroxidase, 40 mU/ml PBS/BR). It was washed again as described above and the filter membrane was incubated for 1 min in a 1:100 mixture of detection reagent A+B (Boehringer Mannheim) after removing the buffer with an absorbent cloth, and adhering reagent was removed. Subsequently an X-ray film was exposed for 1 - 10 min with the filters covered with a household foil.

Result

Using a ca. 10-fold lower antibody concentration, it is possible to detect a 30-fold lower amount of GST-HA using the antibodies according to the invention such as clone 3F10 compared to known antibodies such as clone 12CA5 (Fig. 2).

3.2 Variation of the concentration of the primary antibody

4 ng of the glutathione-S-transferase modified with the HA epitope (GST-HA) was separated in each lane by means of SDS polyacrylamide gel electrophoresis and, after transfer onto a nylon membrane, reacted with the stated

antibodies in the stated concentrations of the primary antibodies (clone 12CA5 and 3F10; 2.0 - 0.008 ng/ml). Bound anti-HA antibodies were subsequently detected using anti-mouse-peroxidase (in the case of clone 12CA5) and anti-rat-peroxidase (in the case of clone 3F10) and by chemiluminescence detection.

SDS polyacrylamide gel electrophoresis, transfer onto a nylon membrane and subsequent detection were carried out as described in example 3.1.

Result:

In the described experiment clone 12CA5 (prior art) becomes the limiting factor at an antibody concentration of $\leq$ 2000 ng/ml whereas at an antibody concentration of ca. 125 ng/ml signal saturation is observed with the clone 3F10 according to the invention (Fig. 3).

Comparable signals are obtained in the case of clone 3F10 at ca. 20-fold lower antibody concentrations.

Example 4

Comparable immunoblot analysis of a HA-modified protein using antibody conjugates

Glutathione-S-transferase modified with the HA epitope (GST-HA) was serially diluted to the stated amounts, separated by means of SDS polyacrylamide gel electrophoresis and, after transfer onto a nylon membrane, reacted with the aid of the stated antibody-peroxidase conjugates at the stated concentrations (100 ng/ml

(clone 12CA5); 20 mU/ml (clone 3F10)). Bound anti-HA antibodies were subsequently detected using chemiluminescence detection.

SDS polyacrylamide gel electrophoresis, transfer onto a nylon membrane and subsequent detection were carried out as described in example 3.1.

Result:

The detection limit in this system for the peroxidase conjugate of the clone 12CA5 (prior art) is ca. 1 ng GST-HA whereas 40 pg GST-HA can still be detected under the same conditions with the corresponding derivative of the clone 3F10 (Fig. 4).

Example 5

Comparative immunoprecipitation of a HA-modified protein

Green-fluorescent protein modified with the HA epitope (GFP-HA) was reacted with the stated amounts [in µg] of anti-HA antibodies (clone 12CA5 and clone 3F10) and immunoprecipitated after adding protein-G-agarose. The precipitates obtained were solubilized, separated by means of SDS polyacrylamide gel electrophoresis and, after transfer onto a nylon membrane, detected with an anti-HA-peroxidase conjugate (clone 12CA5) and by chemiluminescence detection.

Immunoprecipitation

50 ng of the green-fluorescent protein modified with the

HA epitope (GFP-HA) was diluted in 200 µl wash buffer, the stated amounts of anti-HA antibody were added and it was incubated for 1 h at 4°C in an overhead shaker. 25 µl of a 50 % protein-G-Sepharose suspension was then added to the mixture and it was again incubated for 1 h as before. Proteins bound to the gel matrix via protein G were washed three times with 1 ml wash buffer and in each wash step the mixture was incubated for 2 min at 4°C as described above. The mixture was then centrifuged for 2 min in a bench centrifuge (15,000 rpm, RT) and the pellet was taken up in 10 ml 1 x Laemmli application buffer. The immunoprecipitated proteins were subsequently separated by denaturing gel electrophoresis (SDS polyacrylamide gel).

SDS polyacrylamide gel electrophoresis, transfer onto a nylon membrane and subsequent detection were carried out as described in example 3.1.

Result:

Compared to the clone 12CA5 (prior art) the clone 3F10 is able in the described experiment to precipitate the antigen used with a ≥ 20-fold lower antibody quantity (Fig. 5).

INTERNATIONAL FORM

Boehringer Mannheim GmbH
Sandhofer Str. 116

68305 Mannheim

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: R3A12	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2286

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I. above was accompanied by:

( ) a scientific description
( ) a proposed taxonomic designation

(Mark with a cross where applicable).

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 1996-10-08 (Date of the original deposit)[1].

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion).

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wirths Date: 1996-10-18

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 0196

INTERNATIONAL FORM

Boehringer Mannheim GmbH
Sandhofer Str. 116

68305 Mannheim

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Boehringer Mannheim GmbH Sandhofer Str. 116 Address: 68305 Mannheim	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM ACC2286 Date of the deposit or the transfer[1]: 1996-10-08

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 1996-10-09 [2].
On that date, the said microorganism was

(X)[3] viable

( )[3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH Address: Mascheroder Weg 1b D-38124 Braunschweig	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): U. Wacko Date: 1996-10-18

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
[2] In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
[3] Mark with a cross the applicable box.
[4] Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 0196

Claims

1. Monoclonal antibodies against the epitope YPYDVPDYA which is derived from the haemagglutinin of the human influenza virus, or fragments thereof, wherein they have an affinity of > 10^8 M^{-1}.

2. Monoclonal antibodies as claimed in claim 1, wherein they have an affinity of 10^9 - 10^{10} M^{-1}.

3. Monoclonal antibodies as claimed in one of the claims 1 or 2, wherein they are produced by hybridomas which are obtained by fusing mouse P3x63-Ag8.653 myeloma cells with B lymphocytes from Lou/C rats where the Lou/C rats were immunized with a HA peptide.

4. Monoclonal antibodies as claimed in claim 3, wherein the immunization is carried out with a HA peptide coupled to keyhole limpet haemocyanin (KLH).

5. Monoclonal antibodies as claimed in one of the claims 1 to 4, wherein they are produced by the hybridoma R 3A12 deposited at the "Deutsche Sammlung für Mikroorganismen und Zellkulturen" under the No. DSM ACC2286 (08.10.1996).

6. Process for the production of monoclonal antibodies as claimed in one of the claims 1 to 5, wherein a HA peptide is synthesized and it is used to

immunize small mammals, the B lymphocytes are isolated from the spleen of the animals and fused with mouse P3x63-Ag8.653 myeloma cells, the clones that are formed which bind to a HA peptide and to a HA fusion protein are selected and the clones with a high affinity are selected from these and established as hybrid cell lines.

7. Process as claimed in claim 6, wherein the acetyl-**YPYDVPDYA**GSGSK (ε-biotinoyl) amide or biotinoyl-ε-Aca-SGSG**YPYDVPDYA** amide is used as the HA peptide.

8. Process as claimed in claim 6 or 7, wherein HA-tagged glutathione-S-transferase is used as the HA fusion protein.

9. Use of monoclonal antibodies as claimed in one of the claims 1 to 5, wherein they are used to detect and isolate native haemagglutinin of the human influenza virus, modified haemagglutinin or HA fusion proteins.

Abstract

The invention concerns monoclonal antibodies against the epitope YPYDVPDYA which is derived from the haemagglutinin of the human influenza virus and are suitable for the detection and isolation of native haemagglutinin of the human influenza virus, of modified haemagglutinin or of haemagglutinin fusion proteins and have an affinity of > 10^8 M^{-1}, in particular of 10^9 to 10^{10} M^{-1}.

Figure 1

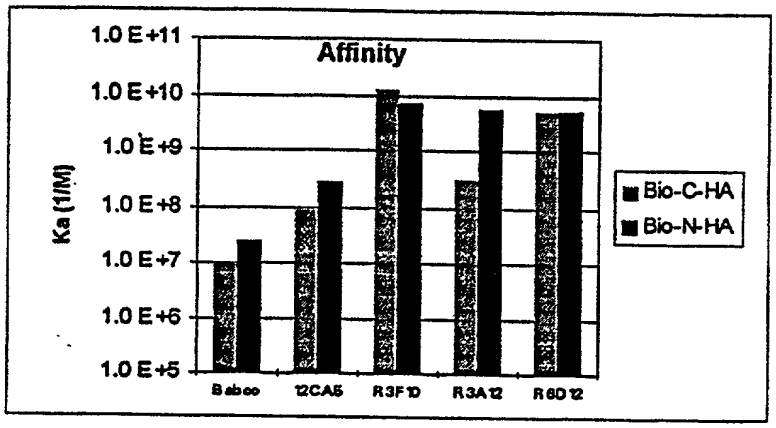


Figure 2

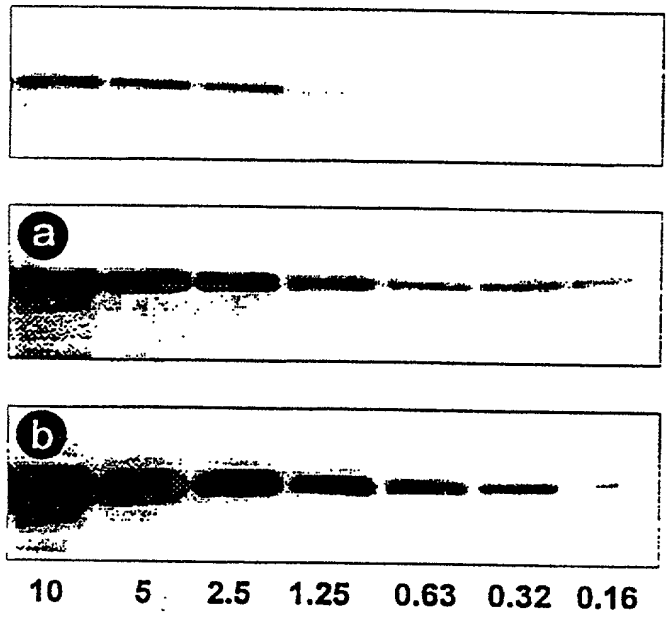
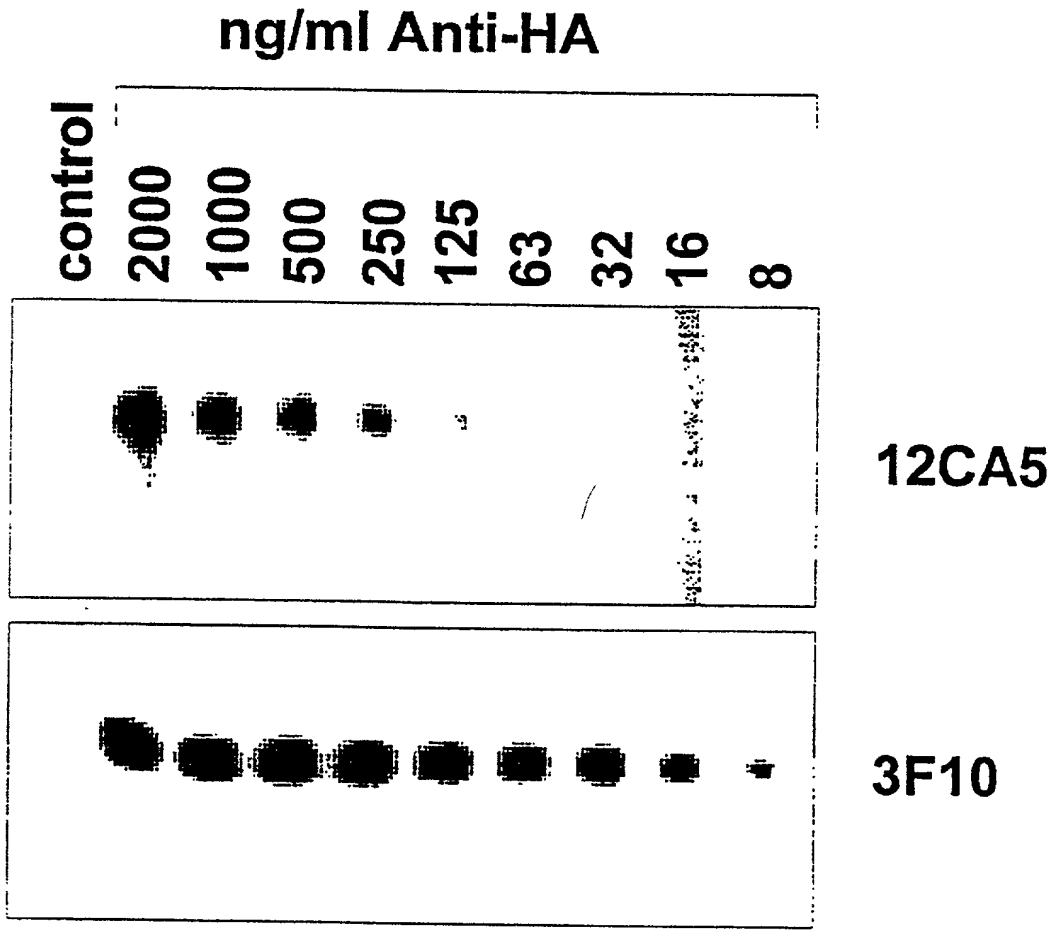


Figure 3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control
2000
1000
500
250
125
63
32
16
8
12CA5
3F10

Figure 4

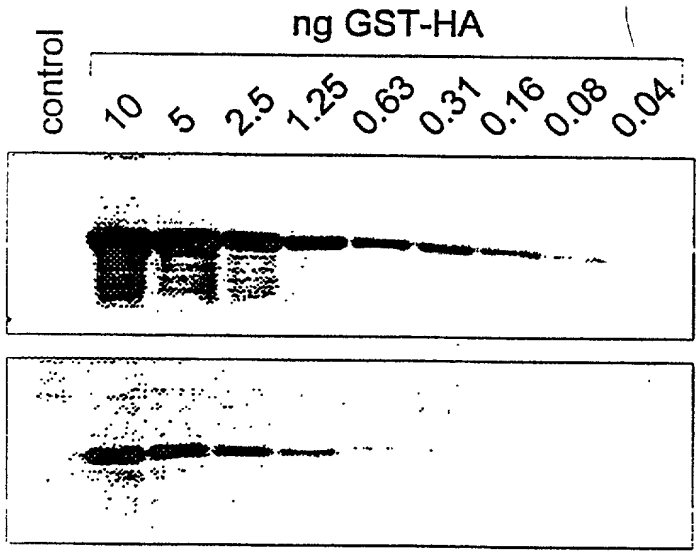
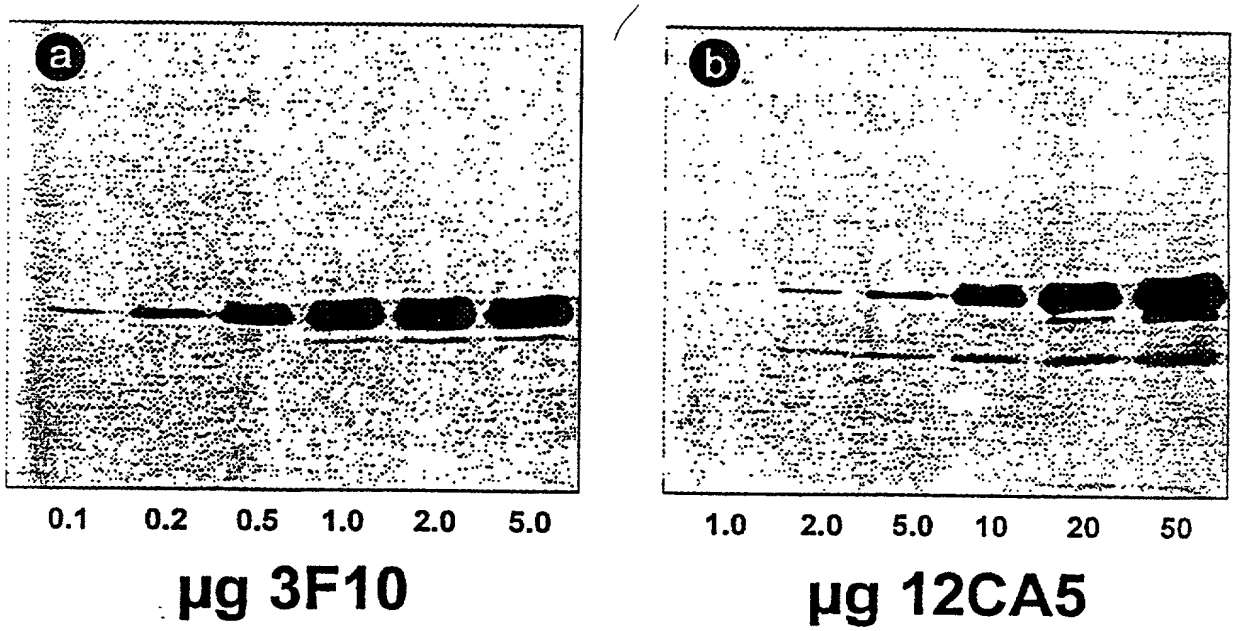


Figure 5



#3

Practitioner's Docket No. BMID9913US *PATENT*

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION, OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is for a national stage of PCT application serial no. PCT/EP97/05783, filed October 20, 1997.

INVENTORSHIP IDENTIFICATION

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

MONOCLONAL ANTIBODIES AGAINST THE EPITOPE YPYDVPDYA, A PROCESS FOR THEIR PRODUCTION AND THEIR USE

SPECIFICATION IDENTIFICATION

The specification of which was filed on April 20, 1999, as U.S. Application Serial No. 09/284,787.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, § 1.56, and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent.

PRIORITY CLAIM (35 U.S.C. § 119(a)-(d))

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international

Such applications have been filed as follows.

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. § 120

I hereby claim the benefit, under Title 35, United States Code, § 120, of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37, Code of Federal Regulations, § 1.56 and that is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent, that occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application. (37 CFR 1.63(e)).

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 USC § 120:				
U.S. APPLICATIONS		Status *(check one)*		
U.S. APPLICATIONS	U.S. FILING DATE	Patented	Pending	Abandoned
PCT APPLICATION DESIGNATING THE U.S.				
PCT APPLICATION NO.	PCT FILING DATE	U.S. APPLICATION NOS. ASSIGNED *(If any)*		
PCT/EP97/05783	20 October 1997			

35 USC § 119 PRIORITY CLAIM, IF ANY,
FOR ABOVE LISTED U.S./PCT APPLICATIONS

ABOVE APPLICATION NO.	DETAILS OF FOREIGN APPLICATION FROM WHICH PRIORITY CLAIMED UNDER 35 USC § 119		
	Country and Application No.	Date of filing (day, month, year)	Date of issue (day, month, year)
PCT/EP97/05783	Germany - 196 43 314.2	21 October 1996	

ALL FOREIGN APPLICATION(S), *IF ANY,* FILED MORE THAN 12 MONTHS
(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

PCT/EP97/05783, filed 20 October 1997
German Patent Application Serial No. 196 43 314.2, filed 21 October 1996

POWER OF ATTORNEY

I hereby appoint the following practitioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Marilyn L. Amick	Registration Number 30,444
D. Michael Young	Registration Number 33,819
Brent A. Harris	Registration Number 39,215
Richard T. Knauer	Registration Number 35,575

AUTHORIZATION OF ATTORNEY(S) TO ACCEPT AND
FOLLOW INSTRUCTIONS FROM REPRESENTATIVE

The undersigned to this declaration and power of practitioner hereby authorizes the U.S. practitioner(s) named herein to accept and follow instructions from

Roche Diagnostics Corporation
9115 Hague Road
Indianapolis, IN 46250-0457

as to any actions to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. practitioner(s) and the undersigned. In the event of a change in the person(s) from whom instructions may be taken, the U.S. practitioner(s) will be so notified by the undersigned.

SEND CORRESPONDENCE TO

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DIRECT TELEPHONE CALLS TO:

Marilyn L. Amick
(317) 576-7561

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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over →

3-00

Michael GROL

Inventor's signature Michael Grol

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